

Enzyme-Linked Immunosorbent Assay with Conserved Immunoreactive Glycoproteins gp36 and gp19 Has Enhanced Sensitivity and Provides Species-Specific Immunodiagnosis of *Ehrlichia canis* Infection[▽]

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Ehrlichia canis is the primary etiologic agent of canine monocytic ehrlichiosis, a globally distributed and potentially fatal disease of dogs. We previously reported on the identification of two conserved major immunoreactive antigens, gp36 and gp19, which are the first proteins to elicit an *E. canis*-specific antibody response, and gp200 and p28, which elicit strong antibody responses later in the acute phase of the infection. In this report, the sensitivities and specificities of five recombinant *E. canis* proteins for the immunodiagnosis of *E. canis* infection by an enzyme-linked immunosorbent assay (ELISA) were evaluated. Recombinant polypeptides gp36, gp19, and gp200 (N and C termini) exhibited 100% sensitivity and specificity for immunodiagnosis by the recombinant glycoprotein ELISA compared with the results obtained by an indirect fluorescent-antibody assay (IFA) for the detection of antibodies in dogs that were naturally infected with *E. canis*. Moreover, the enhanced sensitivities of gp36 and gp19 for immunodiagnosis by the recombinant glycoprotein ELISA compared to those obtained by IFA were demonstrated with dogs experimentally infected with *E. canis*, in which antibodies were detected as much as 2 weeks earlier, on day 14 postinoculation. gp36 and gp19 were not cross-reactive with antibodies in sera from *E. chaffeensis*-infected dogs and thus provided species-specific serologic discrimination between *E. canis* and *E. chaffeensis* infections. This is the first demonstration of the improved detection capability of the recombinant protein technology compared to the capability of the “gold standard” IFA and may eliminate the remaining obstacles associated with the immunodiagnosis of *E. canis* infections, including species-specific identification and the lack of sensitivity associated with low antibody titers early in the acute phase of the infection.

Canine monocytic ehrlichiosis (CME) is a globally distributed, debilitating, and sometimes fatal rickettsial tick-borne disease caused by the obligately intracellular bacterium *Ehrlichia canis* (35). Acute *E. canis* infections are characterized clinically by anorexia, fever, weight loss, depression, lethargy, anterior uveitis, and retinal disease (17). A subclinical phase, absent of clinical manifestations, can develop and persist for years in untreated or inappropriately treated dogs (6). Furthermore, these dogs may develop chronic severe disease, in which the prognosis becomes less favorable and deaths occur from secondary infection and uncontrollable bleeding (35). Other monocytic ehrlichiae, such as *Ehrlichia chaffeensis*, infect dogs, but the clinical manifestations have not been firmly established (3, 9, 44). Canine granulocytic ehrlichiosis, caused by *Ehrlichia ewingii*, manifests as two clinically distinct disease syndromes: chronic, moderate to severe anemia and polyarthritis (7, 14, 33).

The early diagnosis of *E. canis* infection during the acute phase ensures the best prognosis and usually leads to complete

recovery (35). Currently, the indirect fluorescent-antibody assay (IFA) is considered the “gold standard” and is the most widely used method for the diagnosis of CME (37). However, considerations and limitations of IFA include the cross-reactive antibodies generated among related organisms, which can confound the test results; the difficulty in distinguishing a specific etiologic agent; the low-throughput format; the need for a high level of technical expertise to produce the test antigen; and a lack of procedural standardization. Furthermore, IFA requires expensive microscopy equipment, its results are subjectively interpreted, and it is susceptible to inter- and intra-laboratory variation, all of which limit its reliability and applicability, particularly as a point-of-care diagnostic test.

The known serologic cross-reactivity among the numerous agents related to *E. canis* makes the diagnosis of CME by IFA challenging. *E. canis* infections are associated with a poorer prognosis than infection with other cross-reactive ehrlichiae. Moreover, the possibility of ehrlichial coinfections and differences in responses to treatment with antibiotics among animals with *E. canis*, *E. chaffeensis*, and *E. ewingii* infections is becoming better recognized (3, 14, 16). In addition, the fact that dogs may serve as natural reservoirs for *E. chaffeensis* (3, 8) and may be a potential source of human infection demonstrates that species-specific diagnosis could be beneficial to public health. Therefore, diagnostics capable of rapid identification of the

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specific etiologic agent would be advantageous for clinical diagnosis and evaluation of prognosis and treatment and would provide important epidemiologic information. Molecular diagnostics that detect and discriminate medically important *Ehrlichia* spp. now exist (11, 32), but the development of a diagnostic test that can serologically distinguish specific ehrlichial agents has not been reported.

Eight major immunoreactive proteins of *E. canis* have been identified by Western immunoblotting (20); and the majority have been molecularly characterized in *E. chaffeensis*, including p28/30, gp140, gp200, gp36, and gp19 (12, 21–25, 28, 31, 42), as well as genetically divergent orthologs (p28, gp120, gp200, gp47 and VLPT, respectively) (12, 21, 23, 29, 31, 34, 40, 43). *E. canis* gp36 and gp19 elicit the earliest antibody responses and exhibit species-specific antibody reactivity, which has also been demonstrated with gp200 (N terminus, P43) and gp140 (12, 21, 22, 23). Many of these major immunoreactive proteins appear to be useful immunodiagnostic antigens, including p28/p30, gp140, gp200, and MAP2 (2, 22, 25, 28); and a commercially available enzyme-linked immunosorbent assay (ELISA) for the diagnosis of CME based on antibody reactivity to two *E. canis* p30 peptides has been developed (26). This test (Snap 3Dx) has a high specificity compared with the results of IFA when sera from obtained from naturally and experimentally *E. canis* infected dogs are used, but its sensitivity with sera containing low antibody titers (<320) remains questionable (2, 27). An ELISA with recombinant *E. canis* MAP2 has demonstrated high degrees of sensitivity and specificity, but neither of these ELISAs is able to distinguish infections caused by *E. canis* and *E. chaffeensis* (2).

In this study, we evaluated the sensitivities and specificities of five recombinant *E. canis* immunoreactive proteins (p28, gp200 N-terminal protein [gp200N], gp200 C-terminal protein [gp200C], gp36, and gp19) for detection of *E. canis* antibodies using an ELISA. We compared the previously described p28 and gp200N with the newly identified and characterized gp36, gp19, and gp200C. Our results demonstrate that gp36 and gp19 are species-specific antigens that can distinguish infections caused by *E. canis* and *E. chaffeensis*, and they are more sensitive than IFA or other *E. canis* antigens (p28 and gp200) for detection of early acute-phase antibodies.

MATERIALS AND METHODS

Experimental animals. The dogs and the protocols used for the experimental *E. canis* infections were described previously (20). For experimental *E. chaffeensis* infections, two 1-year-old healthy beagle dogs were obtained from a commercial source and housed at the University of Texas Medical Branch Laboratory Animal Resources facility, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Prior to the study, the dogs were demonstrated to lack abnormalities on physical examination and to have no detectable antibodies to *E. chaffeensis* by IFA. The experimental protocol was approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

***E. chaffeensis* and *E. canis* inocula.** The tissue culture infectious dose (TCID₅₀) of the *E. chaffeensis* inoculum was determined by inoculation of DH82 cell monolayers plated in 24-well tissue culture plates with 10-fold dilutions (10^{-1} to 10^{-5}) of inoculum (0.2 ml) in minimal essential medium. The inoculum was incubated for 1 h at 37°C, followed by the addition of 1 ml of growth medium. Seven days after inoculation, the TCID₅₀ was determined by identification of *E. chaffeensis* in inoculated cells by IFA. The TCID₅₀ of the *E. canis* inoculum was determined as described previously (13).

Experimental *E. canis* and *E. chaffeensis* infections in dogs. Two dogs were experimentally infected with *E. chaffeensis* (Arkansas strain) propagated in a

mouse embryo cell line, as described previously (4). Infected cells from six T-150 flasks were harvested by centrifugation at $13,000 \times g$ for 25 min, after the cells were 80% infected. Two dogs received 4 ml of *E. chaffeensis* cell suspension intravenously immediately after preparation, and the 50% TCID₅₀ was determined retrospectively. Immune serum was collected 4 weeks after inoculation, and anti-*E. chaffeensis* and anti-*E. canis* antibody titers were determined by IFA. Fifteen dogs were experimentally infected with *E. canis*, and serum was collected at weekly intervals, as described previously (20).

Dog sera. Serum samples from ill dogs exhibiting clinical signs or hematologic abnormalities consistent with CME were submitted to the Louisiana Veterinary Medical Diagnostic Laboratory from veterinarians statewide, as described previously (22). Sera (diluted 1:40) were screened by IFA and were separated into groups as *Ehrlichia*-positive and -negative sera. Sera from healthy dogs were obtained from 1-year-old healthy beagle dogs from a commercial breeder (Marshall Farms).

Cloning of the genes of *E. canis* recombinant proteins. The gp19 (nucleotides [nt] 7 to 411), gp36 (nt 28 to 816), gp200N (nt 22 to 564), gp200C (nt 3665 to 4188), and p28-3 (nt 82 to 695) genes were cloned into prokaryotic expression vectors as described previously (12, 22–24; K. A. Nethery, C. K. Doyle, X. F. Zhang, and J. W. McBride, submitted for publication). The primers were designed for in-frame insertion of amplicons into the pUni/V5-His-TOPO vector and were recombined with the pBAD Thio-E Echo acceptor vector (Invitrogen Corporation, Carlsbad, CA) (p28) or cloned directly into a pBAD/TOPO Thio-Fusion expression vector (Invitrogen) (gp19, gp36, gp200N, and gp200C).

Expression and purification of *E. canis* recombinant proteins. The gp19, gp36, gp200N, and gp200C recombinant proteins were expressed in *Escherichia coli* (TOP10) after induction with 0.02% arabinose for 2 h. Bacteria (from 10 liters of fermentation culture) were harvested by centrifugation at $5,000 \times g$ for 40 min and resuspended in phosphate-buffered saline (PBS). Recombinant proteins (gp19, gp36, gp200N, and gp200C) were purified under native conditions by lysing the bacteria, which had been resuspended in lysis buffer (PBS, 0.05% Triton X-100, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM imidazole), by sonication and pelleting the insoluble material by centrifugation at $10,000 \times g$ for 1 h. The clarified supernatant was loaded onto an equilibrated Ni-NTA column (50-ml column). The bound recombinant protein was washed with 15 column volumes of increasing concentrations of imidazole (4%, 8%, 20%, and 100%) and eluted with 250 mM imidazole in lysis buffer. Recombinant p28 protein was purified under denaturing conditions by sonicating the pelleted bacteria that had been resuspended in lysis buffer (50 mM Tris-HCl, 400 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) at 50 W for 30 min (20 s on, 20 s off) in ice water and pelleting the insoluble material by centrifugation ($10,000 \times g$) for 30 min. The pellet was washed three times (first with 2 M urea, then with 4 M urea in lysis buffer, and then with water) by stirring the mixture for 30 min at room temperature and was then repelleted (by centrifugation at $6,000 \times g$) for 30 min. The final wash was performed in 4 M urea plus 1% Triton X-100 and 0.1% deoxycholic acid with stirring for 1 h at room temperature, and the protein was pelleted by centrifugation ($10,000 \times g$, 45 min). The pellet was resuspended in sample buffer (4 M urea, 6 M guanidine, 50 mM 2-mercaptoethanol) with overnight stirring at 4°C and pelleted (by centrifugation at $10,000 \times g$, 40 min). The clarified supernatant was loaded onto an equilibrated reversed-phase column (26/10 XK; Amersham Biosciences), washed with buffer A (0.1% trifluoroacetic acid), and eluted with 6 column volumes of increasing ratios (from 0% to 100% buffer B [0.1% trifluoroacetic acid, 85% acetonitrile]) of buffer A and buffer B.

ELISA. The antibody responses to five *E. canis* recombinant proteins (gp36, gp19, p28, gp200N, and gp200C) were evaluated by an ELISA. The ELISA protocol was optimized, including the type of ELISA plate, the protein concentrations, the serum dilutions, and the blocking buffers used. Recombinant gp36 (0.3 µg/ml), gp19 (1.2 µg/ml), p28 (2.5 µg/ml), gp200N (1.4 µg/ml), gp200C (0.5 µg/ml), and thioredoxin as a control (2.5 µg/ml) were diluted in PBS; the assay plate (Nunc-Immuno Plates with a Polysorp surface; Nunc, Roskilde, Denmark) wells were coated with 50 µl containing the recombinant proteins; and the plates were incubated at room temperature for 2 h or overnight at 4°C. The plates were washed four times with 200 µl of wash buffer (PBS and Tween 20, 0.05%), blocked with 100 µl of blocking buffer (10% equine serum in PBS; HyClone Laboratories, Inc., Logan, UT), and incubated for 1 h at 37°C. Each primary antibody was diluted 1:250 in blocking buffer; 50 µl of the antibody was added to duplicate test wells containing antigen, a control well containing recombinant thioredoxin (negative control), and a blank well containing no antigen; and the plates were incubated at room temperature for 1 h. The plates were washed, and 50 µl of affinity-purified peroxidase-labeled goat anti-dog immunoglobulin G (IgG; heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1,000 in blocking buffer was added to each well. The plates were

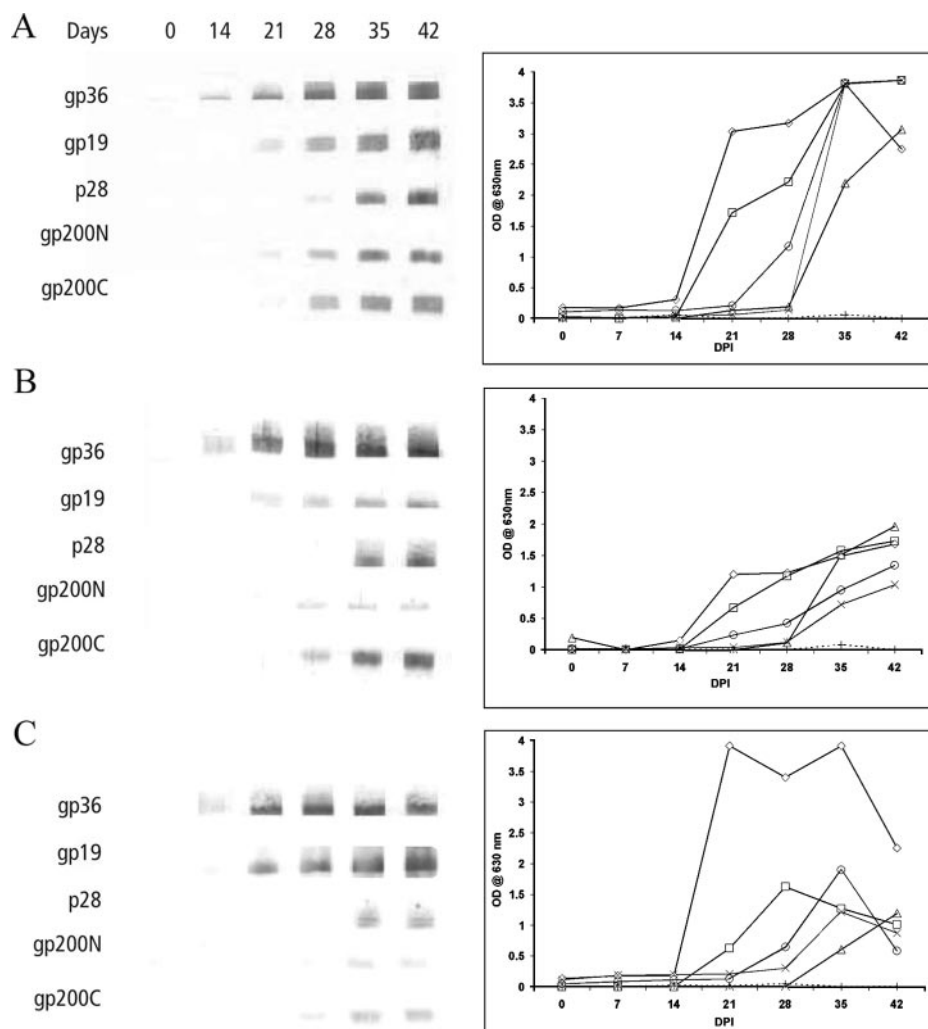


FIG. 1. Analysis of the kinetics of the antibody (IgG) responses in three experimentally infected dogs (A, B, and C) directed at five *E. canis* recombinant proteins (gp36 [\diamond], gp19 [\square], p28 [Δ], gp200N [\times], gp200C [\circ], and a thioresdoxin control [$+$]) at 0, 7, 14, 21, 28, 35, and 42 dpi, as determined by Western immunoblotting (left) and the corresponding ELISA (right).

incubated for 1 h at room temperature and washed. Bound antibody was detected after addition of substrate (100 μ l; Sure Blue Reserve peroxidase substrate, Kirkegaard & Perry Laboratories). The plates were read on a tunable microplate reader (Molecular Devices, Sunnyvale, CA) at A_{650} after incubation at room temperature for 20 min. The final absorbance of each sample was plotted as the optical density at 650 nm (OD_{650}) after subtraction of the absorbance from the negative control well. A sample with a reading 0.2 OD unit above the negative control absorbance was considered positive.

Gel electrophoresis and Western immunoblotting. *E. canis* recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and Western immunoblotting was performed as described previously (20).

IFA. The antibody status of dogs experimentally infected with *E. canis*, clinically ill dogs, and naturally infected dogs was determined as described previously (20). The antibody status of healthy dogs and dogs experimentally and naturally infected with *E. chaffeensis* was performed similarly with *E. canis* (Jake strain) and *E. chaffeensis* (Arkansas strain) antigen slides. Sera were assayed by using twofold dilutions in PBS, starting at 1:64.

RESULTS

Comparison of antibody kinetics against *E. canis* recombinant proteins by Western blotting and ELISA. Antibodies to

the *E. canis* major immunoreactive proteins develop differentially during the acute infection (20). The antibody responses to *E. canis* recombinant proteins in three experimentally infected dogs were examined by ELISA and Western immunoblotting to determine the correlation of the results between the two immunoassays and to determine if the kinetics previously observed with native *E. canis* lysates were reproduced with the recombinant proteins. Antibodies in sera from the three *E. canis*-infected dogs reacted the earliest (day 14) with recombinant gp36 by both Western immunoblotting and ELISA, followed by reactivity with gp19 (day 21). The p28 and gp200 N- and C-terminal polypeptides exhibited similar detection sensitivities, reacting with antibodies later in the course of infection (days 28 to 35), i.e., approximately 2 weeks later than the time that they reacted with gp36 (Fig. 1).

Analytical sensitivity and specificity of *E. canis* recombinant glycoprotein ELISA. The current gold standard for immunodiagnosis is IFA. We used this standard to determine the sensitivity and specificity of our recombinant protein ELISA.

TABLE 1. Analytical sensitivity and specificity of *E. canis* immunodiagnosis with recombinant proteins and IFA

Dog group	No. (%) of dogs with detectable antibodies				
	IFA	gp36	gp19	gp200N	gp200C
Experimentally infected with <i>E. canis</i> (<i>n</i> = 15) ^a	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)
Naturally infected with <i>E. canis</i> (<i>n</i> = 14)	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)
Total (<i>n</i> = 29)	29 (100)	29 (100)	29 (100)	29 (100)	29 (100)
Clinically healthy (<i>n</i> = 10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Clinically ill (<i>n</i> = 26)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (<i>n</i> = 36)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Experimentally infected with <i>E. chaffeensis</i> (<i>n</i> = 2)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Naturally infected with <i>E. chaffeensis</i> (<i>n</i> = 2) ^b	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)

^a Sera were collected at 56 days postinoculation.^b The dogs exhibited at least fourfold higher IgG antibody titers to *E. chaffeensis* antigen by IFA.

Antibodies against recombinant gp36, gp19, gp200N, and gp200C were detected by ELISA in all 29 IFA-positive samples from dogs experimentally infected with *E. canis* (antibody titer range, 1,280 to >10,240) and naturally infected with *E. canis* (antibody titers, $\geq 3,200$ for four dogs, $\leq 1,600$ for four dogs, ≤ 800 for three dogs, and ≤ 400 for three dogs) (Table 1). The recombinant proteins (the gp36, gp19, and gp200 N- and C-terminal polypeptides) exhibited 100% specificity in the ELISA compared to the results of IFA with sera from healthy and ill dogs (Table 1). Conversely, recombinant p28 exhibited high levels of nonspecific antibody binding (above negative control levels) with some dog serum samples and thus had a substantially lower specificity ($\sim 60\%$; data not included in Table 1).

Earlier detection of anti-*E. canis* antibodies with recombinant glycoproteins. Dogs experimentally infected with *E. canis* (*n* = 15), in which antibody response kinetics were defined (20), were used to determine the detection sensitivity of IFA compared to those of the recombinant proteins in the ELISA. The experimentally infected dogs (with one exception) developed IgG antibodies to *E. canis* gp36 that could be detected by ELISA by 14 days postinoculation (dpi), and one-third of these dogs (*n* = 5) had antibodies that reacted with gp19 (Table 2).

TABLE 2. Comparison of serologic sensitivities of detection of *E. canis* infection by IFA and ELISA (gp36 and gp19) in experimentally infected dogs

Dog no.	Detection of <i>E. canis</i> by IFA-gp36 ELISA-gp19 ELISA on day:						
	0	7	14	21	28	35	42
32	—	—	—	—	+	+	+
33	—	—	—	—	+	+	+
34	—	—	—	—	+	+	+
35	—	—	—	—	+	+	+
37	—	—	—	—	+	+	+
41	—	—	—	—	+	+	+
43	—	—	—	—	+	+	+
44	—	—	—	—	+	+	+
45	—	—	—	—	+	+	+
46	—	—	—	—	+	+	+
48	—	—	—	—	+	+	+
51	—	—	—	—	+	+	+
52	—	—	—	—	+	+	+
54	—	—	—	—	+	+	+
59	—	—	—	—	+	+	+

Conversely, none of the dogs had detectable IgG antibodies by IFA at 14 dpi (Table 2). Antibodies were detectable by IFA in only four dogs at 21 dpi. The sensitivities of IFA and the recombinant glycoprotein ELISA became comparable at 28 dpi, but complete agreement was not attained until 42 dpi (Table 2).

Species-specific immunodiagnosis with gp19 and gp36. Four dogs infected with *E. chaffeensis* (two experimentally infected and two naturally infected) were IFA positive for *E. canis* antigen but did not react with the *E. canis* recombinant proteins (gp36, gp19, and gp200) (Table 1). The anti-*E. chaffeensis* antibody titers in the sera from dogs experimentally infected with *E. chaffeensis* were $\geq 1,280$. The anti-*E. chaffeensis* IFA titers of the antibody to the homologous antigen in the sera from dogs naturally infected with *E. chaffeensis* were 1:400 and 1:800 (titer to *E. canis*, 1:64).

DISCUSSION

Early diagnosis of CME in the acute stage of infection, followed by treatment with doxycycline, ensures the best prognosis. Immunodiagnostics capable of providing better sensitivity, particularly during the early acute phase of infection, and the ability to differentiate the specific agent responsible for the infection by use of a well-characterized and consistently reproducible recombinant or synthetic antigen are needed but are unavailable. The recent molecular identification of several distinct but conserved major immunoreactive proteins of *E. canis*, including gp36, gp19, gp200, and p28/30, has created new opportunities for substantial improvements in the serologic diagnosis of CME (12, 21–23, 25, 28). We have previously reported that *E. canis* gp36, gp19, and gp200 are molecularly and immunologically distinct from the respective orthologs in *E. chaffeensis* (gp47, VLPT, and gp200, respectively) and that two of these characterized proteins (gp36 and gp19) are the first to elicit an antibody response in *E. canis*-infected dogs (20). In addition, these proteins are conserved among geographically dispersed *E. canis* strains (10, 23, 24, 25). Therefore, these antigens have a high potential to facilitate the development of an ultrasensitive and highly specific new generation of immunodiagnostics for the detection of *E. canis* infection. We hypothesized that assays with these proteins would provide increased sensitivity over those with whole-cell antigen (IFA) for

the detection of antibodies early in the infection. In this study, we demonstrated that the use of two recombinant *E. canis* proteins (gp36 and gp19) in an ELISA format provided enhanced sensitivity compared to that of IFA for the detection of antibodies during the early immune response and were highly specific for *E. canis*.

The molecularly characterized recombinant proteins (gp36, gp19, p28/30, and gp200) reacted with antibody from infected canine sera with kinetics similar to those reported for the corresponding proteins in native *E. canis* lysates (20) in two immunoassay formats (ELISA and a membrane assay) that are commonly used for point-of-care diagnostic tests. These results confirm that the recombinant proteins are suitable surrogates for native ehrlichial proteins and that they react similarly with antibodies generated during an infection. Furthermore, the consistent results obtained by two immunoassay formats suggest that these proteins could provide consistent sensitivity regardless of the assay format used. In this particular study, Western immunoblotting provided results similar to those of the ELISA; but the results can be laboratory dependent and the Western immunoblotting technique is laborious, time-consuming, and not well suited for point-of-care tests.

The analytical sensitivity of the assay with the *E. canis* recombinant proteins completely correlated with that of the IFA when sera from dogs with natural and experimental infections were used. We previously reported 100% sensitivity with *E. canis* gp200N (P43) (22), and those results were confirmed in this study. However, we have recently identified five major epitopes within the gp200 protein (Nethery et al., submitted). gp200N (P43) contains two major antibody epitopes, as does the carboxy-terminal region (gp200C) (Nethery et al., submitted). The antibody responses to both gp200 recombinant proteins developed later than the responses to gp36 and gp19, but they reacted strongly with antibody in late-acute-phase sera from experimental dogs. These findings were consistent with those of our previous investigations, in which we observed a strong late-acute-phase antibody response to gp200 (20, 22). Antibody to *E. canis* P28 also developed later in the late-acute-phase immune response. We had previously reported similar antibody response kinetics that were consistent with those to both native and recombinant P28 (20). We observed some nonspecific responses to P28 in the ELISA format, but this is likely due to the presence of other, contaminating proteins. P28 is very insoluble, and thus, production of a highly purified recombinant protein is very difficult to achieve. Nevertheless, the results obtained by Western immunoblotting in this study and other studies suggest that highly purified p28/p30 is a specific immunodiagnostic antigen (2, 22, 22).

The first detectable antibodies to *E. canis* are directed at gp36 and gp19 (12, 20). All of the *E. canis* recombinant proteins provided sensitivities similar to that of IFA in naturally infected dogs; however, in the experimentally infected group of dogs, for which the kinetics of the antibody response could be accurately determined, gp36 and gp19 detected antibodies 7 to 14 days earlier than IFA or ELISA with gp200 and p28. To our knowledge, this is the first demonstration that species-specific *E. canis* proteins are more sensitive than whole-cell antigen for detection of the low antibody levels produced during early-acute-phase ehrlichial infections. Many *E. canis* proteins may be suitable for the detection of late-acute-phase antibodies,

and the sensitivities of specific proteins appear to be related to the disease phase. The sensitivities of *E. canis* antigens, such as p28/p30, gp200, and MAP2, for the detection of antibodies appears to be best in a later disease phase, when sera contain medium to high levels of antibody. However, sera with low antibody levels, such as those obtained early in the infection, identify potential limitations of these recombinant antigens and whole-cell antigen (15, 27). This limitation is particularly relevant to sera collected from dogs early in the infection, when antibody levels are low and when an accurate diagnosis is needed but can be the most challenging serologically.

gp36 and gp19 have species-specific serine-rich major epitopes that have been identified and molecularly characterized (12, 23). Likewise, the *E. canis* and *E. chaffeensis* gp200 orthologs are antigenically distinct and have epitopes that have been molecularly characterized (21, 22; Nethery et al., submitted). The major epitopes on gp36, gp19, and gp200 have carbohydrate immunodeterminants that contribute to the immunoreactivities of the epitopes (12, 23; Nethery et al., submitted). These major immunoreactive antigens can discriminate serologically between *E. canis* and its most closely related organism, *E. chaffeensis*, and will enable the development of highly specific assays capable of discrimination of the specific infecting agent. Another major immunoreactive antigen (gp120) of *E. chaffeensis* capable of sensitive species-specific discrimination has also been reported (39–41). Thus, highly defined recombinant antigens that include the major epitopes of *E. canis* gp36 and/or gp19 and *E. chaffeensis* gp120 could be used in the same assay for the specific diagnosis of *E. canis* and *E. chaffeensis* infections.

The reliability of the serologic diagnosis of infections with recombinant or synthetic antigens depends on the lack of antigenic variability of the antigen that is selected. In the case of *E. canis*, many of the major immunoreactive antigens, including gp36, gp19, gp200, and p28, that have the potential to be used for serodiagnosis are highly conserved in geographically distinct isolates (10, 23, 24, 25, 42). Conversely, in the case of *E. chaffeensis* the major immunoreactive antigens exhibit more diversity among different isolates, but the antibody epitope of gp120 appears to be well conserved (5, 10, 30, 38, 42). Moreover, the differential expression of the major outer membrane proteins (p28/p30), which have antigenically distinct hypervariable regions that contain antibody epitopes (1, 18, 19, 25, 28, 29, 36, 43), may also contribute to variations in serologic responses to *E. canis* and *E. chaffeensis*. Thus, we conclude that antigens such as gp36 and gp19, which are encoded by highly conserved single genes, that minimize or eliminate the potential for serologic variability have the best potential for use in the development of globally useful, ultrasensitive, and species-specific immunodiagnostics that overcome these obstacles associated with the serodiagnosis of CME.

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